Table 2 Effects of *Ginkgo biloba* extract (Ginkgo) treatment on EC50 of relaxation produced by acetylcholine in the aortic rings preconstricted with noradrenaline (10^{-7} M) of spontaneously hypertensive rats

Diet	EC50 (nM)
Control	21.5±2.6
Ginkgo 0.05%	16.2±3.4
Ginkgo 0.1%	17.9±2.5
Ginkgo 0.5%	12.6±1.0*

Each value is the mean±s.e.m. for six rats. *$P<0.05$ vs control.

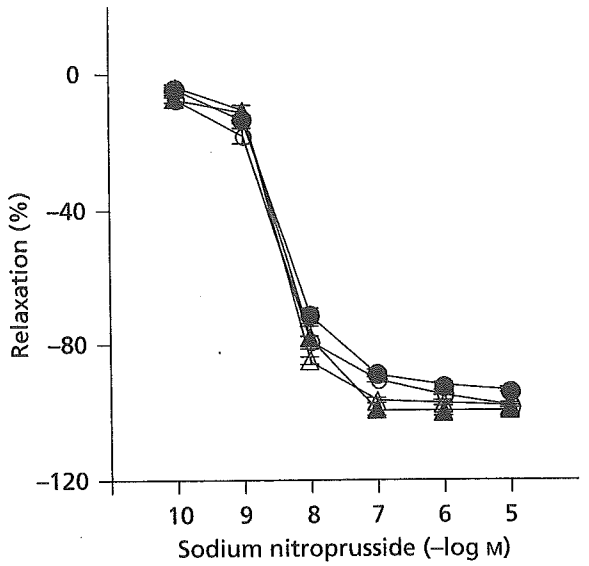

Figure 3 The effect of 0.5% *Ginkgo biloba* extract (Ginkgo) diet on relaxation induced by sodium nitroprusside in aortic rings preconstricted with noradrenaline (10^{-7} M) isolated from normotensive Wistar Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). The ordinate indicates the ratio of relaxation (%) to maximum relaxation in response to papaverine (10^{-6} M) and the abscissa denotes the concentration of sodium nitroprusside (M) in rats fed the control diet (▲, WKY; ●, SHR) or the 0.5% Ginkgo diet (△, WKY; ○, SHR). Each point represents the mean±s.e.m. (n=6).

was restored by long-term administration of a Ginkgo-containing diet. This effect of Ginkgo was not observed in WKY. However, a significant decrease in EC50 values was observed only with 0.5% Ginkgo diet and a significant increase in maximum relaxation values was observed only with 0.1% and 0.5% Ginkgo diets. These results indicated that ACh-induced relaxation at 10^{-7}–10^{-5} M concentrations was enhanced by 0.1% and 0.5% Ginkgo diets in SHR. Furthermore, relaxation in response to nitric oxide, such as sodium nitroprusside-induced relaxation, was not affected by a Ginkgo-containing diet in either SHR or WKY. These findings suggested that enhanced relaxation resulting from Ginkgo administration was due to increased nitric oxide production/release from the endothelium or to greater nitric oxide bioavailability.

Flavonoids are considered important dietary antioxidants (Robak & Gryglewski 1988). Ginkgo also has antioxidant properties (Haramaki et al 1994; Koc et al 1995; Miyajima et al 1997; Pietri et al 1997). Sasaki et al (2002) reported that Ginkgo produced antioxidant effects, increased nitric oxide metabolites, and increased the expression of endothelial nitric oxide synthase mRNA in stroke-prone SHR. One of the major active ingredients of Ginkgo is considered to be quercetin. Indeed, quercetin was reported to inhibit hypoxanthine-xanthine oxidase activity and scavenge superoxides, hydroxy radicals, and peroxynitrite in-vitro (Rice-Evans & Packer 1998). Moreover, metabolites of orally administered quercetin have been demonstrated to retain the antioxidant properties of the parent compound (Manach et al 1998). Duarte et al (2001a, b) demonstrated that while quercetin reduced elevated blood pressure and restored the endothelium-dependent vasodilation in response to ACh in SHR, no such effects were apparent in WKY. They suggested that the effects of quercetin were associated with a reduced oxidant status due to its antioxidant properties. Superoxide is generally recognized to impair endothelium-dependent vasodilation via inactivation of synthesis and/or release of nitric oxide and consequently to elevate blood pressure (Taniyama & Griendling 2003). Taken together, these findings suggested that the antioxidant properties of the flavonoids within Ginkgo, such as quercetin, may have participated in the effects of Ginkgo on blood pressure and endothelium-dependent relaxation observed in this study.

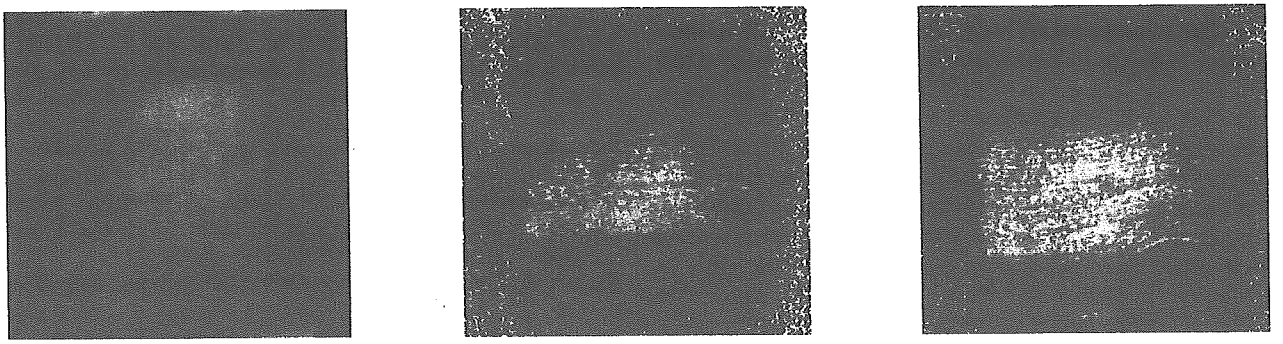

Figure 4 The effect of acetylcholine on intracellular calcium ion level in aortic endothelium isolated from spontaneously hypertensive rats (SHR). Left panel: phase-contrast microscopic image of the endothelial layer. Stainless steel wires are identified in the top and bottom of the image. The degree of vasoconstriction can be estimated from the distance between these two wires. The remaining panels show fluorescent confocal images of intracellular calcium ion level in the endothelial layer of the aorta precontracted with noradrenaline (10^{-6} M) in the absence (middle panel) and presence (right panel) of acetylcholine (10^{-6} M).

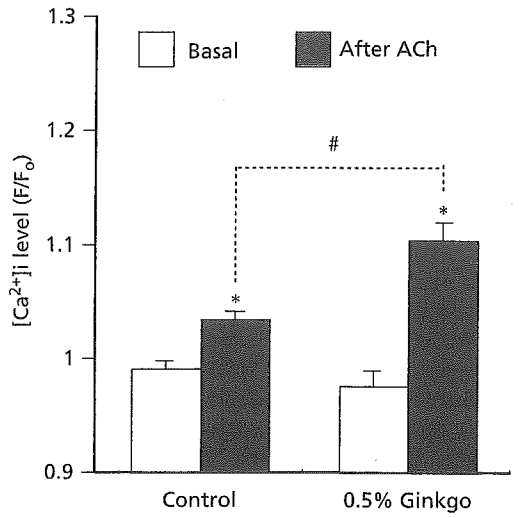

Figure 5 The effects of 0.5% *Ginkgo biloba* extract (Ginkgo) diet on the intracellular calcium ion increase induced by acetylcholine (ACh) in the aortic endothelium isolated from spontaneously hypertensive rats (SHR) fed with control and 0.5% Ginkgo diets. The ordinate shows the intracellular calcium ion level (F/F_0) (fluorescence intensity was the peak fluorescence intensity (F) divided by fluorescence intensity at the beginning of each experiment (F_0)). Each column represents the mean ± s.e.m. (n = 6). $^*P < 0.05$ vs each baseline value. $^{\#}P < 0.05$ vs control acetylcholine.

Indirect and direct experimental evidence demonstrated that the entry of extracellular Ca^{2+} and the liberation of Ca^{2+} from intracellular stores could contribute to an increase in free cytoplasmic Ca^{2+} concentration in endothelial cells, which was an essential step in the synthesis and release of nitric oxide (Rubanyi & Vanhoutte 1988). Endothelial nitric oxide synthase is constitutively expressed in endothelial cells lining the blood vessels and heart. Its activity is tightly controlled by an intramolecular auto-inhibitory element that hinders calmodulin binding and this molecular hindrance is removed by elevated intracellular Ca^{2+} levels (Wu et al 2002; Fleming & Busse 2003). Thus, many studies have indicated that the release of nitric oxide must require an increase in cytoplasmic Ca^{2+} within endothelial cells. Pogan et al (2001) have shown that the Ca^{2+} signalling process in SHR endothelial cells was affected by increased oxidative stress, resulting in a depletion of releasable Ca^{2+} from inositol 1,4,5-trisphosphate-sensitive and -insensitive Ca^{2+} pools. They suggested a possible beneficial action of antioxidants on Ca^{2+} signalling in endothelial cells from models of hypertension. In our study, a greater ACh-induced increase in intracellular Ca^{2+} level was observed in endothelial cells of the aorta isolated from SHR that had been fed a Ginkgo-containing diet than in those isolated from control SHR. This accelerated response may have been caused by recovery of an intracellular Ca^{2+} mobilization mechanism in endothelial cells resulting from the antioxidative action of Ginkgo flavonoids. However, further study is necessary to elucidate the site of action and mechanism of Ginkgo.

In conclusion, the results confirmed that Ginkgo reduced the elevation of blood pressure and improved the dysfunction of the endothelial nitric oxide synthase/nitric oxide pathway in the endothelium in SHR. Ginkgo enhanced the increase in intracellular Ca^{2+} level to ACh in endothelial cells. This acceleratory influence of Ginkgo on intracellular Ca^{2+} mobilization may have participated in the restoration of endothelium-dependent vasodilation in response to ACh. To our knowledge, this was the first study to have investigated the influence of long-term Gingko administration on intracellular calcium mobilization in vascular endothelium. The pharmacological activity was considered to contribute to the possible beneficial properties of Ginkgo in clinical practice, including the regulation of hypertension.

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Effects of *Ginkgo Biloba* Extract Feeding on Salt-Induced Hypertensive Dahl Rats

Yoko KUBOTA,[a,b] Naoko TANAKA,[a] Satomi KAGOTA,[a] Kazuki NAKAMURA,[a] Masaru KUNITOMO,[a,b] Keizo UMEGAKI,[c] and Kazumasa SHINOZUKA*,[a]

[a] *Department of Pharmacology, School of Pharmaceutical Sciences, Mukogawa Women's University;* [b] *Research Institute for Biosciences, Mukogawa Women's University; Nishinomiya 663–8179, Japan: and* [c] *National Institute of Health and Nutrition; Shinjuku-ku 162–8636, Japan.* Received August 22, 2005; accepted November 16, 2005

We previously demonstrated that *Ginkgo biloba* extract (GBE) produced vasodilation *via* the nitric oxide synthesis and release by increasing the intracellular calcium level in vascular endothelial cells of rats. The present study aimed to clarify the effects of dietary administration of GBE on the blood pressure and vascular tone of hypertensive Dahl salt-sensitive (Dahl) rats in order to evaluate its therapeutic actions and availability. Dahl rats were fed an 8.0% NaCl diet or an 8.0% NaCl plus 0.5% GBE diet for 24 d. The feeding of GBE did not change the heart rate, but significantly decreased systolic blood pressure. After 24 days' administration, the effects of GBE on the atria and aorta isolated from Dahl rats were examined. The GBE-containing diet did not affect the negative and positive actions of isolated atria that were produced by acetylcholine and isoproterenol, respectively. In the aortic preparations, the relaxation in response to acetylcholine was significantly potentiated by a GBE-containing diet. Sodium nitroprusside-induced relaxation was unchanged by GBE-containing diet. These results demonstrated that GBE reduced salt-related elevation of blood pressure and restored the impaired acetylcholine-induced vasodilation in aortic segments.

Key words *Ginkgo biloba* extract (GBE); Dahl salt-sensitive rat; blood pressure; aorta; relaxation

Ginkgo biloba extract (GBE), which is the leaf extract of *Ginkgo biloba*, has many pharmacological effects. For example, preventing ischemia-induced oxidation,[1—3] improving cerebral blood flow[4] and antagonizing the action of platelet-activating factor[5] have been reported. GBE and its constituents, especially terpenoids and flavonoids, are also reported to possess vasorelaxant properties.[6,7] These findings have led us to consider the possibility that GBE might have protective effects in cardiovascular disease. However, few reports have clarified the effect of GBE on blood pressure, using an animal model of hypertension. Dahl salt-sensitive (Dahl) rats develop high blood pressure when fed salt, and are therefore similar to a subgroup of humans with hypertension.[8,9] In this study, we analyzed the effects of daily-term oral GBE treatment on blood pressure, heart and vascular function in Dahl rats.

MATERIALS AND METHODS

Animals and Materials Experiments were performed in accordance with Guiding Principles for the Care & Use of Laboratory Animals approved by The Japanese Pharmacological Society and Mukogawa Women's University. The GBE powder was supplied by Tama Biochemical Co., Ltd. (Tokyo, Japan) and contained 24.2% flavonoids and 9.4% terpenes; similar to that of EGb 761®[10] used in European countries. Male 6-week-old Dahl salt-sensitive rats ($n=12$) were obtained from Japan SLC, Inc. (Shizuoka, Japan). Rats were initially fed a control diet (8% NaCl addition MR stock (Japan SLC, Inc.) diet without GBE) for 7 d. After that, the animals received 8% NaCl addition MR stock diet (control group, $n=6$) or 8% NaCl plus 0.5% GBE addition MR stock diet (GBE group, $n=6$) for 24 d. The animals had free access to drinking water in the experiment. Rats were then anaesthetized with pentobarbital sodium (60 mg/kg, i.p.), blood was taken from the abdominal aorta, and the heart and thoracic aorta were rapidly removed. Heart rate and blood pressure were measured by the tail-cuff method (Model MK-2000, Muromachi Kikai Co., Ltd., Tokyo, Japan) in unanaesthetized rats between 13.00 and 17.00 h, at 23—25 °C.

Materials Acetylcholine chloride, *R*(−)-isoproterenol (+)-bitartrate salt and noradrenalin were obtained from Daiichi Pharmaceutical Co. (Tokyo, Japan), RBI (Natick, MA, U.S.A.) and Sankyo Co. (Tokyo, Japan). Other reagents used were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Chronotropic and Inotropic Effects on Isolated Rat Atria The isolated heart was immediately placed in a Krebs–Henseleit solution of the following composition (mM): NaCl 118.4, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, KH_2PO_4 1.2 and glucose 10.0. After excess blood vessels and the ventricle were removed from the heart, the atria preparation which consists of auricular nodes and left-right atrium were mounted in a 5 ml organ bath filled with Krebs–Henseleit solution. The chronotropic and inotropic changes were measured with a force-displacement transducer (Model T-7, NEC San-ei Instruments, Ltd., Tokyo, Japan) coupled to a PowerLab/800 (ADInstruments Pty Ltd., NSW, Australia) under a resting tension of 1.0 g. The bath solution was maintained at 32 °C to avoid the exhaustion condition of the atria and bubbled with a 95% O_2–5% CO_2 gas mixture. Each preparation was allowed to equilibrate for at least 60 min prior to initiation of experimental procedures, and during this period the incubation medium was changed every 20 min. After the equilibration period, acetylcholine or isoproterenol were cumulatively added to the bath solution.

Relaxation Studies on Isolated Rat Aorta The thoracic aorta was immediately placed in a Krebs–Henseleit solution of the following composition (mM): NaCl 118.4, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, KH_2PO_4 1.2 and glucose 10.0. After removing periaortic fat and connective tissue, the aorta was cut into ring segments of approximately 3 mm length. Each ring preparation was mounted vertically

* To whom correspondence should be addressed. e-mail: kazumasa@mwu.mukogawa-u.ac.jp

under a resting tension of 1 g in a 5 ml water jacketed organ bath filled with Krebs–Henseleit solution and attached to a force-displacement transducer (Model T-7, NEC San-ei Instruments, Ltd., Tokyo, Japan). The bath solution was maintained at 37 °C and bubbled with a 95% O_2–5% CO_2 gas mixture. Each preparation was allowed to equilibrate for at least 60 min prior to the initiation of experimental procedures, and during this period the incubation media were changed every 10 min. After this equilibration period, the ring preparation was contracted with noradrenaline (10^{-7} M) before cumulatively adding putative relaxing agents. The relaxation response was expressed as a percentage of the maximal relaxation developed by papaverine (10^{-4} M).

Statistics All values are presented as the means±S.E.M. Data were evaluated for statistical significance using the Student's *t*-test. When the variances of two groups were different, the Welch test was used. A probability of less than 0.05 was considered significant. Statistical analyses were carried out with a computer program (StatView 5.0, SAS Institute Inc., Cary, NC, U.S.A.).

RESULTS

Effect of GBE Diet on Blood Pressure and Heart Rate Figure 1 shows systolic blood pressure and heart rate in Dahl rats during 24 days' administration of control or GBE-containing diets. Systolic blood pressure in control Dahl rats significantly increased with age (after days 0, 8, 16 and 24 the systolic blood pressures were 129.2±5.1, 142.3±2.9, 158.6±2.3 and 166.3±3.6 mmHg, respectively). This increase was significantly suppressed by administration of GBE (after days 0, 8, 16 and 24 the systolic blood pressures were 132.9±4.3, 120.5±3.0, 130.6±2.8 and 145.5±3.5 mmHg, respectively). However, GBE-containing diet did not affect heart rate over the 24 d administration period.

Effect of GBE Diet on Isolated Rat Atria Figure 2 compares the effects of acetylcholine and isoproterenol on the atria isolated from Dahl rats receiving GBE-diet with those from Dahl rats receiving the control diet. The heart rate and contractile force in atria isolated from Dahl rats receiving GBE diet were not significantly different from those from control rats. Acetylcholine-induced negative chronotropic and inotropic actions in atria of GBE rats were not significantly different from those of control rats. Also, isoproterenol-induced positive chronotropic and inotropic effects in atria of GBE rats were not significantly different from those of control rats.

Effect of GBE Diet on Isolated Rat Aorta Figure 3 shows the influence of GBE-containing diet on relaxation induced by acetylcholine and sodium nitropurusside in the aortic rings pre-contracted with noradrenaline (10^{-7} M). In the aorta of Dahl rats fed GBE-containing diet for 24 d, the maximum relaxation by acetylcholine (87.0±2.4%) was significantly larger than that observed in Dahl rats fed the control diet (74.3±5.3%). Sodium nitropurusside-induced relaxation was not changed after 24 days' administration of GBE-diet. The contractile force with noradrenaline (10^{-7} M) was not significantly different after 24 d of GBE-containing diet (0.63±0.03 g) compared with control diet (0.69±0.03 g).

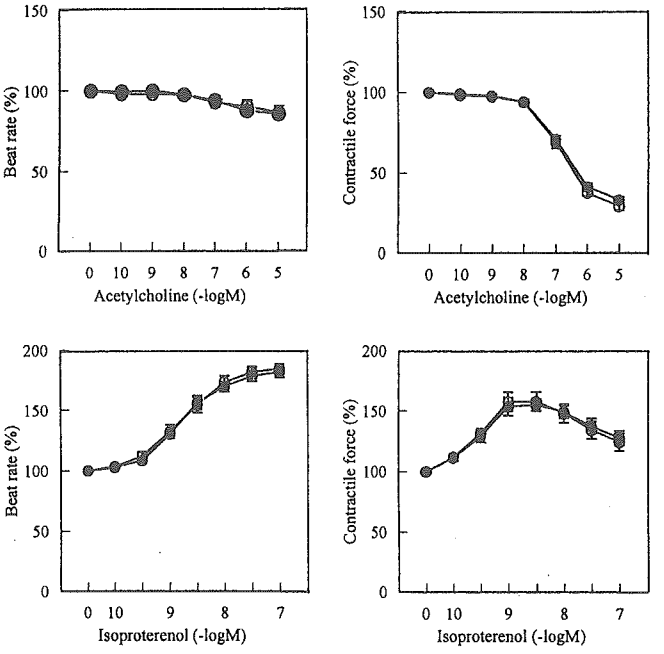

Fig. 2. The Effects of 0.5% *Ginkgo biloba* Extract (GBE) Diet on Chronotropic and Inotropic Actions Induced by Acethylcholine and Isoproterenol in the Atria Isolated from Dahl Rats

The ordinate denotes the ratio of beat rate (left) and contractile force (right), and the abscissa indicates the concentration of acethylcholine (upper) and isoproterenol (lower) after control (●) and GBE (○) diets. Each point represents the mean±S.E.M. (n=6).

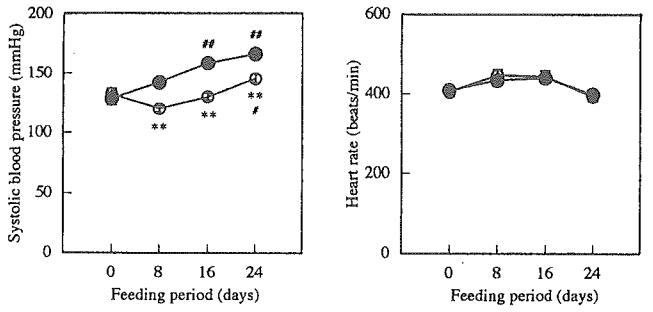

Fig. 1. Effects of 0.5% *Ginkgo biloba* Extract (GBE) Diet on Systolic Blood Pressure and Heart Rate in Dahl Rats

The ordinate denotes systolic blood pressure (left) and heart rate (right), while the abscissa indicates the time course (d) after control (●) and 0.5% GBE (○) diets. Each point represents the mean±S.E.M. (n=6). $^{**}p<0.01$ *vs.* control. $^{\#,\#\#}p<0.05$, 0.01 *vs.* each control (0 d).

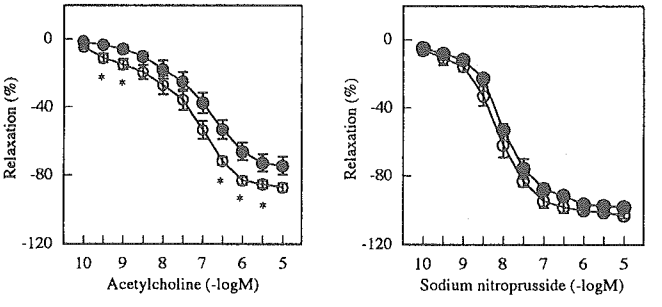

Fig. 3. The Effect of 0.5% *Ginkgo biloba* Extract (GBE) Diet on Relaxation Induced by Acethylcholine and Sodium Nitropurusside in the Aortic Rings Preconstricted with Noradrenaline (10^{-7} M) Isolated from Dahl Rats

The ordinate denotes the ratio of relaxation (%) to maximum relaxation in response to papaverine at 10^{-4} M and the abscissa indicates the concentration of acethylcholine (left) and sodium nitropurusside (right) after control (●) and GBE (○) diets. Each point represents the mean±S.E.M. (n=6). $^{*}p<0.05$ *vs.* control.

DISCUSSION

GBE contains approximately 30 kinds of flavonoids and their derivatives, plus terpenoids such as ginkgolide A, ginkgolide B, ginkgolide C and bilobalide.[10] Our previous study[11] demonstrated that GBE produced dose-dependent vasodilation *via* nitric oxide (NO) synthesis and release by increasing the intracellular calcium level in vascular endothelial cells of rats. We have also suggested that one of the principal ingredients of GBE for bringing about vasodilation is quercetin, which has already been reported to exert antihypertensive effects in spontaneously hypertensive rats (SHR) when administered orally on a long-term basis.[1,2] Sasaki *et al.*[12] have shown that the age-related increase in blood pressure observed in SHR was suppressed significantly by GBE at 60—120 mg/kg each day for 3 weeks. Umegaki *et al.*[13] have already found that GBE produced antihypertensive effects in deoxycorticosterone acetate-salt hypertensive rats. In the present study, 24 days' administration of GBE caused a significant hypotensive effect in Dahl rats. It is well known that vascular resistance is regulated by the endothelium *via* the synthesis and secretion of a variety of vasoactive substances, such as nitric oxide, prostacycline, endothelium-derived hyperpolarizing factors (EDHF) and endothelium-derived contracting factor (EDCF). The stable balance of these factors released from the endothelium is disturbed in diseases such as hypertension, atherosclerosis, and diabetes. In hypertension, the endothelium-dependent relaxation induced by a variety of vasodilator agents, such as acetylcholine, is markedly impaired, and this has been documented repeatedly by various investigators.[14—18] The overproduction of vasoconstrictor prostanoids[14,19] and super oxide anions generated in this pathologic process have been proposed as factors that contribute to the impaired relaxation of vessels to endothelium-dependent vasodilators.[15,20] Akpaffiong and Taylor[21] have also suggested that either excess production of oxidants or deficiency of antioxidant systems may contribute to high blood pressure and vascular endothelial impairment in SHR. Recently, Sasaki *et al.*[12] reported that the anti-oxidant effects of GBE were increased by measurement of urinary 8-hydroxy-2′-deoxyguanosine.

In our *in vitro* experiments, relaxation of aortae isolated from Dahl rats in response to acetylcholine was potentiated by long term administration of a GBE-containing diet. Relaxation in response to NO, *e.g.* sodium nitropurusside-induced relaxation was not affected by treatment with GBE in Dahl. In the aorta of Dahl rats fed on a high-sodium diet, endothelium-dependent relaxations in response to various vasodilators are impaired, while the relaxations in response to the endothelium-independent agonist, sodium nitropurusside, are only slightly impaired.[22,23] These findings suggest that enhanced relaxation resulting from GBE administration is due to increased or restored NO production/release from the endothelium or to greater NO bioavailability.

Flavonoids are considered important dietary antioxidants.[24] GBE also has antioxidant properties[1—3,25] and one of its major active ingredients is considered to be quercetin. Indeed, quercetin was reported to inhibit hypoxanthine-xanthine oxidase activity and scavenge super oxide, hydroxyl radicals, and peroxynitrite *in vitro*.[26] Moreover, metabolites of quercetin after oral administration have also been found to retain the antioxidant properties of the parent compound.[27] Superoxide is generally recognized to impair endothelium-dependent vasodilation *via* inactivation of synthesis and/or release of NO and consequently to elevate blood pressure.[28] Taken together, these findings suggest that the antioxidant properties of the flavonoids within GBE, such as quercetin, may participate in the effects of GBE on blood pressure and endothelium-dependent relaxation observed in the present study. However, further study will be necessary to elucidate the site of action and mechanism of GBE.

In conclusion, the present data demonstrate that GBE reduces the elevation of blood pressure and improves the dysfunction of the NO pathway in the endothelium of Dahl rats. These pharmacological activities are considered to contribute to the possible beneficial properties of GBE in clinical practice, including the regulation of hypertension.

Acknowledgements This study was supported in part by a grant from the Ministry of Health, Labor and Welfare of Japan.

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Disturbances in nitric oxide/cyclic guanosine monophosphate system in SHR/NDmcr-cp rats, a model of metabolic syndrome

Satomi Kagota *, Yu Yamaguchi, Naoko Tanaka, Yoko Kubota, Kyoko Kobayashi, Namie Nejime, Kazuki Nakamura, Masaru Kunitomo, Kazumasa Shinozuka

Department of Pharmacology, School of Pharmaceutical Sciences, Mukogawa Women's University, Nishinomiya, Japan

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Abstract

Metabolic syndrome is a cluster of metabolic abnormalities, including hypertension, hyperlipidemia, hyperinsulinemia, glucose intolerance and obesity. In such lifestyle-related diseases, impairment of nitric oxide (NO) production or bioactivity has been reported to lead to the development of atherogenic vascular diseases. Therefore, in the present study we investigated changes in the NO/cyclic guanosine monophosphate (cGMP) system in aortas of SHR/NDmcr-cp (*cp/cp*) rats (SHR-cp), a model of the metabolic syndrome. In aortas of SHR-cp, endothelium-dependent relaxations induced by acetylcholine and endothelium-independent relaxations induced by sodium nitroprusside were significantly impaired in comparison with Wistar-Kyoto rats. Furthermore, protein levels of soluble guanylyl cyclase and cGMP levels induced by sodium nitroprusside were significantly decreased. In contrast, protein levels of endothelium NO synthase and cGMP levels induced by acetylcholine were significantly increased, and plasma NO_2 plus NO_3 levels were also increased. The levels of lipid peroxide in plasma and the contents of 3-nitrotyrosine, a biomarker of peroxynitrite, in aortas were markedly increased. These findings indicate that in the aortas of SHR-cp, NO production from the endothelium is augmented, although the NO-induced relaxation response is impaired. Enhanced NO production may be a compensatory response to a variety of factors, including increases in oxidative stress.

Keywords: Endothelium; Metabolic syndrome; Nitric oxide; Vascular smooth muscle; Vasorelaxation

Introduction

The vascular endothelium, the thin cell layer that covers the inner wall of blood vessels, regulates the tonus of underlying smooth muscle cells by releasing vasorelaxing factors such as nitric oxide (NO) (Furchgott and Vanhoutte, 1989). NO causes vasorelaxation via the NO/cyclic guanosine monophosphate (cGMP) pathway, which is the essential pathway of the relaxation response in the vascular system (Murad, 1986; Hanafy et al., 2001). NO also has an important role of protecting against atherogenesis by causing vasodilation and inhibiting growth and migration of vascular smooth muscle cells, platelet aggregation and thrombosis, monocyte adhesion, inflammation, and lipoprotein oxidation (Maxwell et al., 1998). Therefore, impairment of NO production or bioactivity promotes the development of atherosclerosis (Schulz et al., 2004).

Metabolic syndrome is a cluster of metabolic abnormalities, including obesity, hyperinsulinemia, hyperglycemia, hyperlipidemia, and hypertension, with underlying insulin resistance. The coexistence of these disorders has been increasingly linked with atherogenic vascular diseases (Reaven, 1993; Lteif and Mather, 2004; Hsueh et al., 2004). There has been increasing evidence of a parallel progression between insulin resistance and endothelial dysfunction (Hsueh et al., 2004; Lteif and Mather, 2004). For instance, impairment of endothelium-dependent relaxations has been reported in insulin resistant subjects, with the development of obesity, hypertension and hyperlipidemia (Steinberg et al., 1996), in animal models of metabolic syndrome (Zanchi et al., 1995; McNamee et al., 1994; Karagiannis et al., 2003). In contrast, unchanged or even enhanced endothelium-dependent relaxations have been reported in obese Zucker rats, an animal model of metabolic syndrome, which develop insulin resistance associated with

* Corresponding author. 11-68 Koshien Kyuban-cho, Nishinomiya 663-8179, Japan. Tel./fax: +81 798 45 9944.
E-mail address: skagota@mwu.mukogawa-u.ac.jp (S. Kagota).

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marked hyperinsulinemia, dyslipidemia and hypertension (Auguet et al., 1989; Sexl et al., 1995; Subramanian and MacLeod, 2003). In addition, a recent study has demonstrated that endothelial NO synthase (eNOS) expression and post-translation regulation do not diminish in the obese Zucker rats (Fulton et al., 2004). Furthermore, it is known that in the pathophysiology of atherosclerosis, hypertension and diabetes, the upregulation eNOS often represents a compensatory mechanism accompanying increased production of superoxide and other reactive oxygen species (Li et al., 2002b). Thus, vascular responses may change with animal species and age. Therefore, the present study was aimed at elucidating whether or not vasorelaxation responses via the NO production of the endothelium actually deteriorate in the metabolic syndrome. We investigated the NO production and vasorelaxation response via the NO/cGMP pathway in thoracic aortas isolated from obese spontaneously hypertensive rats, SHR/NDmcr-cp (*cp/cp*) rats (SHR-cp) in comparison with their lean littermates, SHR/NDmcr-cp (+/+) rats (SHR) and normotensive Wistar-Kyoto rats (WKY). The SHR-cp is an inbred subline of SHR/N-corpulent rats (SHR/N-cp), which have the corpulent (*cp*) gene (Koletsky, 1975), and has been shown to spontaneously develop obesity, hypertension, hyperlipidemia, hyperglycemia, and hyperinsulinemia, the so-called metabolic syndrome.

Materials and methods

Experimental animals

Male SHR-cp, SHR and WKY established by the Disease Model Cooperative Research Association (Hamamatsu, Japan) at 7, 18, 23 and 36 weeks of age ($n=6$ for each age group) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). In another experiment, male SHR-cp, SHR and WKY at 18–23 weeks of age ($n=3-6$ for each age group) were used. Rats of each strain were maintained on a standard diet and given water ad libitum in an air-conditioned room. The systolic blood pressure was determined in conscious rats by the indirect tail-cuff method (MK-2000, Muromachi, Tokyo, Japan). The study protocols were performed according to the Guideline Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society.

Relaxation studies

Thoracic aortas were removed and immediately placed in Krebs–Henseleit solution (118.4 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2{\cdot}2H_2O$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4{\cdot}7H_2O$, 25.0 mM $NaHCO_3$, 11.1 mM glucose). The vessels were cleaned of their adherent tissue and cut into 3-mm rings, taking care not to damage the endothelium. The rings were mounted isometrically at an optimal resting tension of 1.0 g in a 10-mL organ bath filled with the solution (37 °C, pH 7.4) described above. The bath solution was continuously aerated with a gas mixture of 95% O_2 and 5% CO_2 and the rings were then allowed to equilibrate for 60 min before the start of the experiments. Isometric tension change was measured with a force-displacement transducer (Model t-7, NEC San-Ei, Tokyo, Japan) coupled to a dual channel chart recorder (Model 8K21, NEC San-Ei). The aortic rings were preconstricted with 0.1–0.3 μM phenylepherine to generate approximately 80% of the maximal contraction. Once a stable contraction was obtained, acetylcholine (0.1 nM–1 μM), sodium nitroprusside (0.1 nM–1 μM), nitroglycerin (0.1 nM–1 μM) or 8-(4-chloro-phenylthio)-cGMP (8pCPT-cGMP, 0.01–100 μM) were cumulatively added to the bath. The relaxation response obtained was expressed as a percentage of the maximal relaxation caused by 100 μM papaverine.

Determination of cGMP level

Aortic rings from three strains were removed and placed in Krebs–Henseleit solution bubbled with a gas mixture of 95% O_2 and 5% CO_2. The rings were mounted isometrically at an optimal resting tension of 1.0 g in a 10-mL organ bath filled with the solution (37 °C, pH 7.4) described above. After preincubation for 20 min with 1 μM zaprinast, a cGMP-dependent phosphodiesterase inhibitor, the rings were constricted with phenylepherine (0.1 μM) for 5 min and then stimulated with acetylcholine (0.03 μM) or sodium nitroprusside (0.01 μM) for 1 or 3 min, respectively. The tissues were immediately frozen in liquid nitrogen and then homogenized in a glass/glass homogenizer in ice-cold 6% trichloroacetic acid. The homogenates were centrifuged at 3000 *g* for 15 min at 4 °C. The supernatants were extracted three times in five volumes of ether, and the aqueous phase was lyophilized. The cGMP content was determined with an enzyme immunoassay kit (cGMP enzyme immunoassay Biotrak EIA system, Amersham Biosciences, Buckinghamshire, UK). Protein in the precipitates was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). The amount of cGMP was expressed in picomoles cGMP per milligram protein of the sample.

Western blot analysis

The thoracic aortas were homogenized in a glass/glass homogenizer in lysis buffer [50 mM Tris–HCl buffer (pH 7.5), containing 0.15 M NaCl, 10 mM EDTA, 0.1% Tween-20, 0.01% (v/v) protease inhibitor cocktail (Sigma Chemical Co., St Louis, MI, USA) and 1 mM dithiothreitol]. The protein concentration was determined for each sample using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). An equivalent amount of total arterial protein (20 μg) was loaded on the SDS-PAGE (PAG Mini DAIICHI 6/9, Daiichi Pure Chemicals Co., Ltd. Tokyo, Japan) and then blotted onto nitrocellulose. The membrane was incubated overnight at 4 °C with mouse monoclonal antibodies to endothelium NO synthase (eNOS) (Transduction Laboratories, KY, U.S.A.), inducible NOS (iNOS) (Transduction Laboratories), beta-actin (Sigma Chemical, Co.) or alpha-actin (Progen, Heidelberg, Germany) protein, and with a rabbit antibody to soluble guanylyl cyclase (sGC) protein (Alexis Biochemicals, CA, U.S.A.). The blots were washed, then incubated for 2 h at room temperature with peroxidase-conjugated anti-mouse IgG

Table 1
Body weight, systolic blood pressure and plasma levels of triglyceride, total cholesterol and glucose in WKY, SHR and SHR-cp at 7, 18, 23 and 36 weeks of age

		7 weeks	18 weeks	23 weeks	36 weeks
Body weight (g)	WKY	179±7	364±4	387±5	462±9
	SHR	209±8	358±8	372±6	461±9
	SHR-cp	259±8*,#	479±15*,#	550±13*,#	697±13*,#
Systolic blood pressure (mm Hg)	WKY	123±5	124±3	130±3	134±1
	SHR	143±7*	186±4*	200±4*	211±3*
	SHR-cp	152±3*	167±6*,#	194±7*	217±5*
Triglycerides (mg/100 mL)	WKY	84.4±3.9	72.1±2.6	78.5±4.5	78.2±6.5
	SHR	77.2±3.1	78.7±5.6	94.6±4.9	76.0±3.5
	SHR-cp	205±21*,#	707±82*,#	973±116*,#	796±62*,#
Cholesterol (mg/100 mL)	WKY	87.9±2.0	102±4	105±5	103±5
	SHR	55.4±1.9*	79.2±2.3*	82.1±3.4*	75.0±3.4*
	SHR-cp	101±4*,#	159±8*,#	183±6*,#	149±5*,#
Glucose (mg/100 mL)	WKY	123±8	126±5	127±6	133±9
	SHR	145±7	124±4	152±6	140±6
	SHR-cp	194±18*,#	207±12*,#	231±15*,#	155±3*

Results are expressed as the mean±S.E.M. ($n=6$ for each group). *$P<0.05$, as compared with the WKY group, #$P<0.05$, as compared with the SHR group.

(Vector Laboratories, CA, U.S.A.), or anti-rabbit IgG (Transduction Laboratories). Subsequent detection of the specific proteins was achieved with enhanced chemiluminescence (Western blotting luminal reagent, Santa Cruz Biotechnology Inc., CA, U.S.A.) on X-ray film for 1 to 3 min. The X-ray film was scanned into Adobe Photoshop (Ver. 3.0.5) with a scanner (hp psc 2310 all-in-one, Hewlett–Packard Company, CA, U.S.A.) and transferred to the Macintosh NIH-Image program (Ver. 1.63). The density of the bands was measured using NIH-Image gel macros. The signals were obtained as the eNOS/beta-actin or sGC/alpha-actin ratio.

Determination of glucose, triglycerides, total cholesterol, thiobarbitureic acid reactive substances (TBARS) and NO_2^- plus NO_3^- (NO_x) levels in plasma

Blood was drawn from the abdominal aorta under anesthesia with pentobarbital sodium (60 mg/kg, i.p.) into tubes contain-

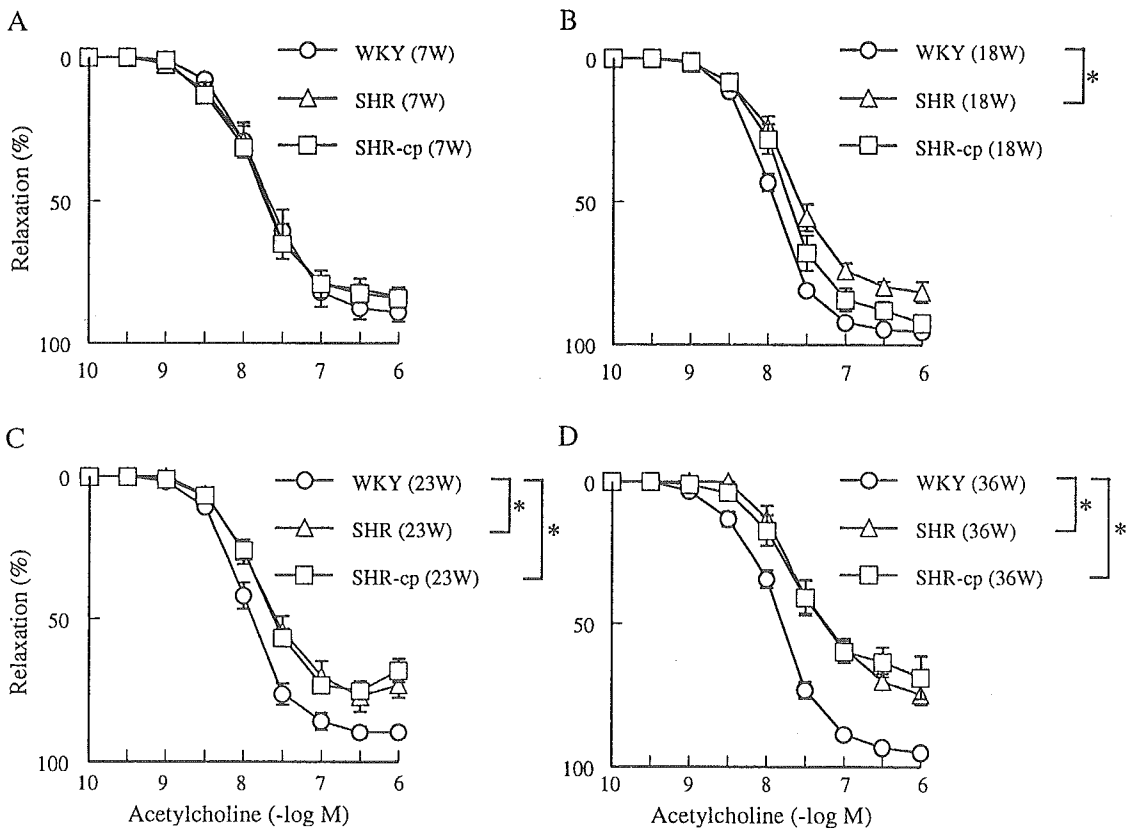

Fig. 1. Changes in endothelium-dependent relaxations in response to acetylcholine in aortic rings from SHR-cp, SHR and WKY at 7 (A), 18 (B), 23 (C) and 36 (D) weeks of age. The relaxation response was expressed as a percentage of the maximal relaxation caused by 100 μM papaverine. Results are expressed as the mean±S.E.M. ($n=6$ for each group). *$P<0.05$, as compared with the WKY.

Table 2
Values of pEC_{50} and R_{max} of relaxation responses to acetylcholine and sodium nitroprusside in thoracic aortas from WKY, SHR and SHR-cp at 7, 18, 23 and 36 weeks of age

	Group	7 weeks	18 weeks	23 weeks	36 weeks
Acetylcholine					
pEC_{50}	WKY	7.85±0.07	7.97±0.03	7.95±0.05	7.87±0.05
	SHR	7.86±0.10	7.74±0.09*	7.79±0.06*	7.54±0.13*
	SHR-cp	7.91±0.05	7.85±0.07	7.80±0.05*	7.60±0.11*
R_{max}	WKY	89.8±3.5	95.4±1.3	91.0±2.2	95.6±0.9
	SHR	85.9±3.0	81.7±3.6*	78.1±5.1*	77.1±4.1*
	SHR-cp	84.8±3.6	92.5±2.6#	77.1±2.6*	68.5±4.2*
Sodium nitroprusside					
pEC_{50}	WKY	8.53±0.10	8.93±0.27	8.55±0.09	8.74±0.08
	SHR	8.47±0.07	8.21±0.07*	8.19±0.06*	8.32±0.04*
	SHR-cp	8.46±0.11	8.19±0.08*	8.09±0.06*	8.28±0.16*
R_{max}	WKY	96.3±1.0	96.4±1.0	95.8±1.1	93.7±2.1
	SHR	90.0±1.6	93.4±2.1	93.3±1.8	89.9±4.3
	SHR-cp	89.5±2.6	94.5±1.2	93.6±1.8	86.2±2.9

Results are expressed as the mean±S.E.M. ($n=6$ for each group). *$P<0.05$, as compared with the WKY group, #$P<0.05$, as compared with the SHR group.

ing EDTA at a final concentration of 1 mg/mL for anticoagulation. The plasma was separated by centrifugation at 3000 *g* for 10 min. Glucose, triglyceride and total cholesterol levels in plasma were determined using commercial kits (Glucose C II-test Wako, Cholesterol E-test Wako, and Triglyceride E-test Wako, respectively, Wako Pure Chemical Ind., Ltd., Osaka, Japan). Lipid peroxide levels in plasma were estimated by assaying TBARS, using the fluorometric method described by Yagi (1976). The plasma NO_2 plus NO_3 (NO_x) levels, as an index of generalized NO production, were determined using a commercial kit (NO_2/NO_3 Assay Kit-C, Dojindo Laboratories, Kumamoto, Japan) based on the Griess reaction.

Determination of 3-nitrotyrosine in aortas

The lipids of each aortic sample were extracted with chloroform:methanol (2:1, v/v), and then the residues were hydrolyzed with 6 M hydrochloric acid for 24 h at 110 °C. After removal of hydrochloric acid from the hydrolysate, the residue was dissolved in distilled water. The determination of 3-nitrotyrosine in aortas was performed by HPLC with coulometric electrochemical array detection as described previously (Yamaguchi et al., 2002).

Drugs

The drugs used in the present experiments were as follows: acetylcholine chloride (Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan); phenylepherine, zaprinast and sodium 8pCPT-cGMP (Sigma Chemical Co., U.S.A.); sodium nitroprusside

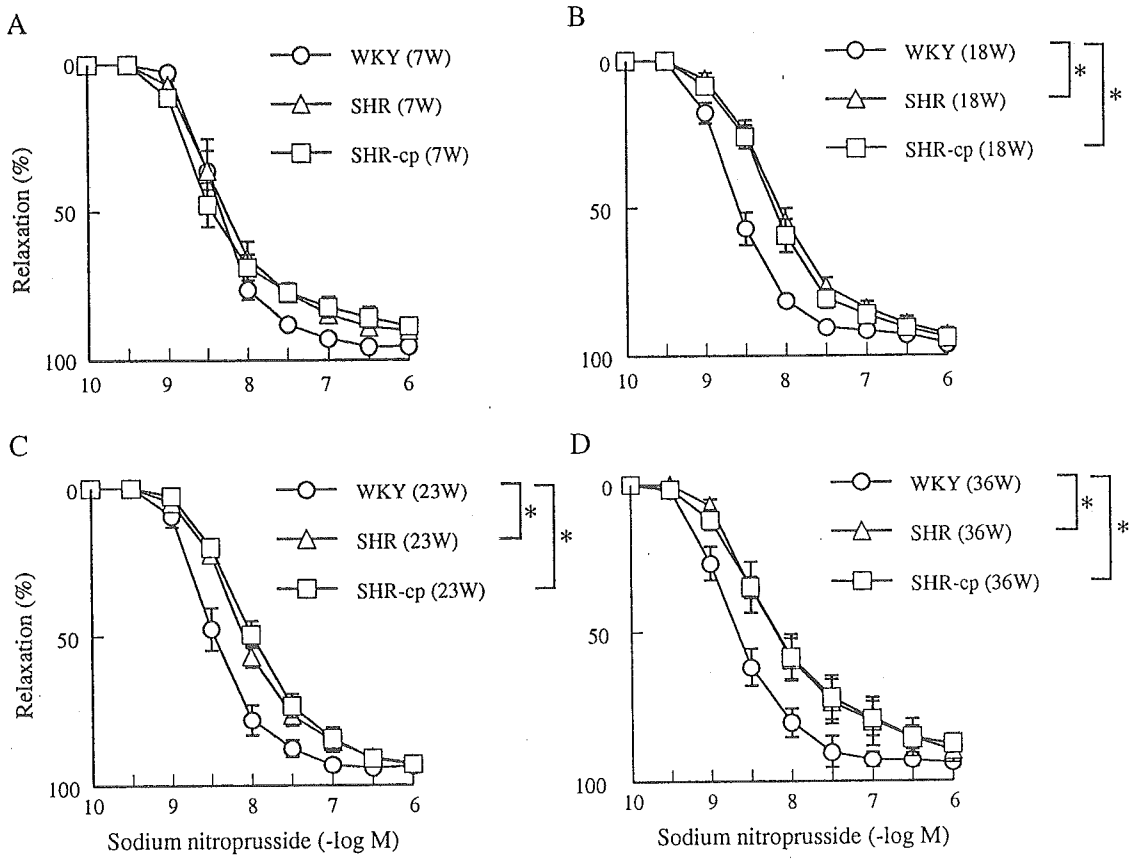

Fig. 2. Changes in endothelium-independent relaxations in response to sodium nitroprusside in aortic rings from SHR-cp, SHR and WKY at 7 (A), 18 (B), 23 (C) and 36 (D) weeks of age. The relaxation response was expressed as a percentage of the maximal relaxation caused by 100 μM papaverine. Results are expressed as the mean±S.E.M. ($n=6$ for each group). *$P<0.05$, as compared with the WKY.

and papaverine hydrochloride (Nacalai Tesque Inc., Kyoto, Japan); nitroglycerin (Millisrol; Nihon Kayaku Co., Ltd., Tokyo, Japan). Other chemicals of analytical reagent grade were purchased from Nacalai Tesque Inc. (Kyoto, Japan). All other compounds were dissolved in distilled water.

Data analysis

Data are expressed as means±S.E.M. Individual concentration–response curves were characterized by determining the pEC_{50} (negative logarithm molar concentration of agonist required to produce 50% of the maximal response) and the R_{max} (maximum relaxation response). The pEC_{50} values and R_{max} were calculated using Graph Pad Prism software (Ver. 4, San Diego, CA, USA). Statistical analysis was performed using the analysis of variance (ANOVA) followed by the Bonferroni–Dann test (Stat View software, Ver. 5.0, SAS, CA, USA). Differences were considered significant at $P<0.05$.

Results

Body weight, blood pressure and plasma lipid and glucose levels

SHR/NDmcr-cp (*cp/cp*) rats (SHR-cp) have been shown to develop spontaneous obesity, hyperinsulinemia, hyperglycemia, hyperlipidemia, and hypertension (Nangaku et al., 2003). In the present study, we also checked body weight, systolic blood pressure levels and plasma triglyceride, cholesterol and glucose levels of the experimental rats at each age.

As shown in Table 1, SHR-cp at 7 weeks of age weighed 145% and 124% more than WKY and SHR, respectively. The increased weight level, approximately 1.4-fold higher than those of WKY and SHR, was observed until 36 weeks of age.

The systolic blood pressure in SHR-cp and SHR was markedly higher in all age groups than that of WKY through the experiment. Only at 18 weeks of age, the systolic blood

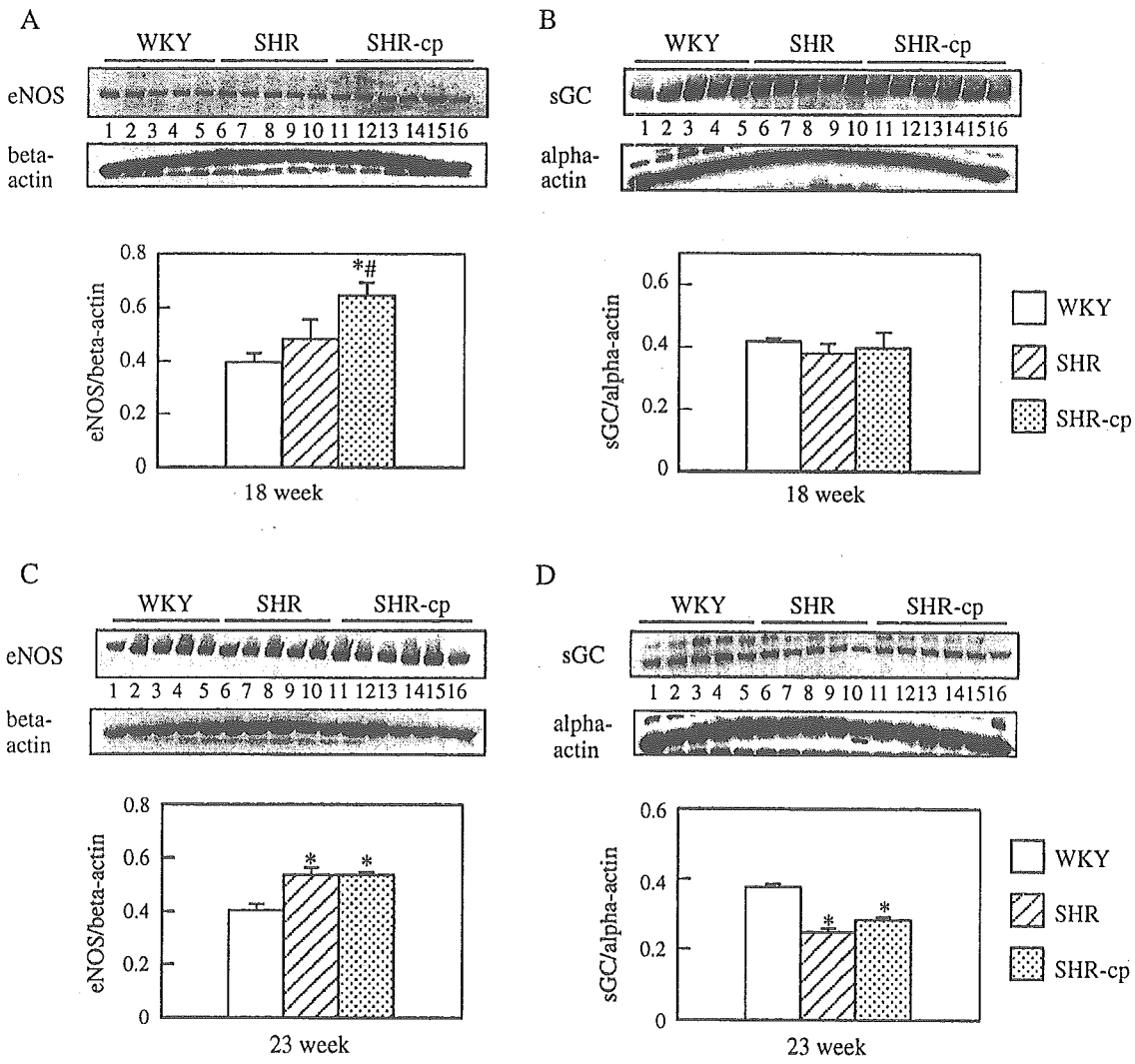

Fig. 3. Changes in protein levels of endothelial nitric oxide synthase (eNOS) (A and C) and soluble guanylyl cyclase (sGC) (B and D) in aortas from SHR-cp, SHR and WKY at 18 (A and B) and 23 (C and D) weeks of age. Lanes 1–5, lanes 6–10 and lanes 11–16 represent the typical pattern of each enzyme protein expression obtained from the aortas of five or six animals in each group. The eNOS protein signals were normalized using the corresponding signals of beta-actin, and the sGC protein signal by that of alpha-actin. The relative protein levels are expressed as the mean±S.E.M. ($n=5–6$ for each group). $^{*}P<0.05$, as compared with the WKY, $^{\#}P<0.05$, as compared with the SHR.

pressures in SHR-cp were slightly but significantly lower than those in SHR. The diastolic blood pressure was also significantly lower in SHR-cp than in SHR at the same age (SHR-cp, 90.5±2.5: SHR group, 129±5 mm Hg). However, there was no significant difference between SHR-cp and SHR at 23 weeks of age (SHR-cp, 134±6: SHR group, 140±9 mm Hg).

The plasma triglyceride levels in SHR-cp were approximately 10-fold higher in all age groups than those of WKY and SHR. The total cholesterol levels in SHR-cp were significantly higher at 7 weeks of age than those of WKY and SHR, and the increment was maintained until 36 weeks of age. The plasma glucose levels in SHR-cp were 1.6-fold and 1.3-fold higher at 7 weeks of age than those in WKY and SHR, respectively, and SHR-cp exhibited hyperglycemia until 36 weeks of age.

Changes in vasorelaxation in aortas

Endothelium-dependent relaxations

In the present study, we chose aortas of SHR-cp to determine changes in the vasorelaxation response via the NO/cGMP pathway in metabolic syndrome, because the endothelium-dependent relaxation in rat aortas is mediated by NO alone. Fig. 1 shows the relaxation in response to acetylcholine in aortic rings isolated from SHR-cp, SHR and WKY at 7, 18, 23 and 36 weeks of age, respectively. Table 2 shows the values of pEC_{50} and the R_{max} of relaxation responses to acetylcholine in thoracic aortas from rats of each strain. The contractile response to phenylepherine, i.e. the level of preconstruction of each ring, was not significantly different among rats of the three strains in all age groups (data not shown).

Acetylcholine-induced endothelium-dependent relaxation was equivalent in rats of three strains at 7 weeks of age (Fig. 1A). At 18 weeks of age, relaxations in response to acetylcholine in the aortic rings of SHR were significantly impaired compared with WKY. However, there was no significant difference in the relaxation between SHR-cp and WKY (Fig. 1B). On the other hand, impaired relaxations in response to acetylcholine were observed in both SHR-cp and SHR at 23 and 36 weeks of age, and the degrees of the impairment were the same (Fig. 1C and D). Similarly, there was no significant difference in endothelium-dependent relaxations in response to adenosine 5′-diphosphate between SHR-cp and WKY at 18 weeks of age, but the relaxations were equally reduced in both SHR-cp and SHR at 23 weeks of age (data not shown).

Endothelium-independent relaxations

Fig. 2 and Table 2 show the endothelium-independent relaxation in response to sodium nitroprusside in aortic rings isolated from SHR-cp, SHR and WKY at 7, 18, 23 and 36 weeks of age. There was no significant difference among the three groups at 7 weeks of age (Fig. 2A and Table 2). At 18 weeks of age, however, the relaxations were impaired in both SHR-cp and SHR compared with WKY, with the degree of impairment being the same between the two strains (Fig. 2B, C and D). Similarly, endothelium-independent relaxations in response to nitroglycerin were equally reduced in both SHR-cp and SHR (data not shown).

Changes in the NO/cGMP pathway in aortas of SHR-cp

Protein levels of eNOS and sGC

Fig. 3 shows the protein levels of eNOS (A and C) and sGC (B and D) in the aorta from rats of three strains at 18 (A and B) and 23 (C and D) weeks of age. The protein levels of eNOS significantly increased by approximately 1.5-fold in SHR-cp compared with SHR and WKY at 18 weeks of age (Fig. 3A), but the degree of the increment was reduced at 23 weeks of age (Fig. 3C). The protein levels in SHR were slightly but significantly increased at 23 weeks of age. On the other hand, the protein levels of sGC did not change in any strains at 18

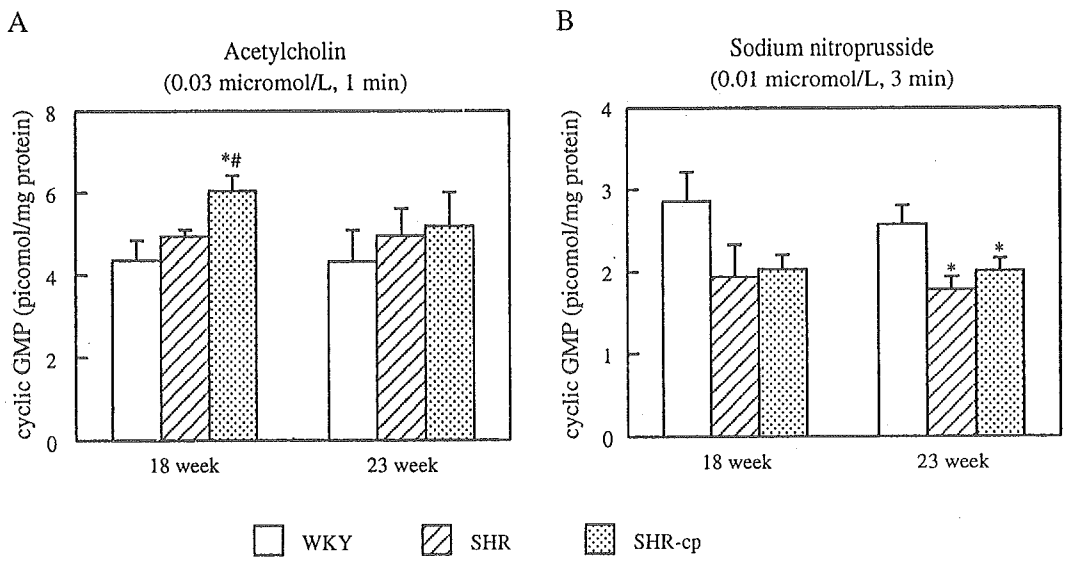

Fig. 4. Changes in cGMP levels induced by acetylcholine (0.03 μmol/L, 1 min) (A) and sodium nitroprusside (0.01 μmol/L, 3 min) in aortic rings from SHR-cp, SHR and WKY at 18 and 23 weeks of age. Results are expressed as the mean±S.E.M. ($n=6$ for each group). $^{*}P<0.05$, as compared with the WKY, $^{\#}P<0.05$, as compared with the SHR.

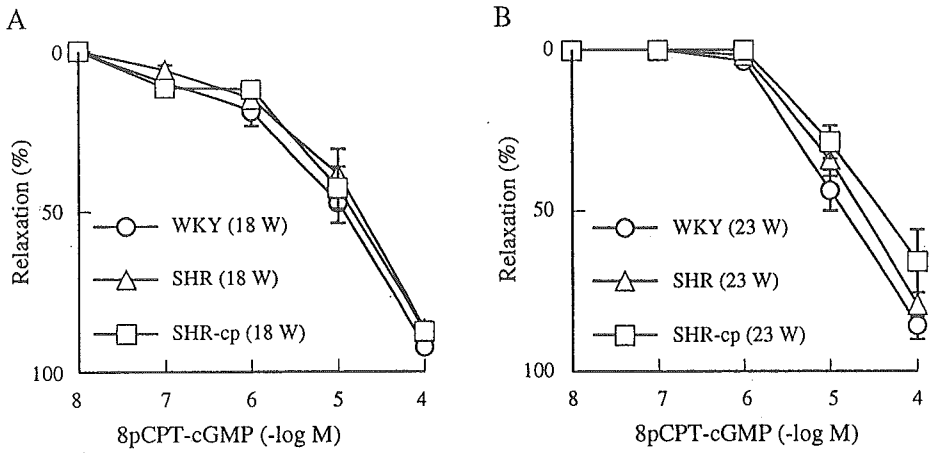

Fig. 5. Changes in relaxations in response to 8pCPT-cGMP, a membrane permeable stable analog of cGMP, in aortic rings from SHR-cp, SHR and WKY at 18 (A) and 23 (B) weeks of age. Results are expressed as the mean±S.E.M. ($n=6$ for each group).

weeks of age (Fig. 3B), and significantly decreased in SHR-cp and SHR compared with WKY at 23 weeks of age (Fig. 3D).

Production of cGMP

As shown in Fig. 4A, the cGMP levels induced by acetylcholine significantly increased in SHR-cp compared with those in WKY and SHR at 18 weeks of age. The levels in SHR-cp at 23 weeks of age showed a tendency to increase. On the other hand, the cGMP levels induced by sodium nitroprusside in both SHR-cp and SHR decreased at 18 and 23 weeks of age compared with WKY (Fig. 4B). However, the levels in SHR-cp were not significantly different from those in WKY at 18 weeks of age.

Relaxation in response to cGMP

To investigate the effect of endogenous cGMP on the relaxation response in smooth muscle cells, we examined relaxation in response to membrane-permeable stable analogs of cGMP, 8pCPT-cGMP in the aorta from three strains at 18 (Fig. 5A) and 23 (Fig. 5B) weeks of age. The 8pCPT-cGMP-induced relaxation was not significantly different among the three strains at 18 weeks of age. At 23 weeks of age, the relaxation in SHR-cp showed a tendency to decrease compared with that in WKY.

NO_x and TBARS levels in plasma

We determined the plasma levels of NO_x and TBARS, as an index of generalized NO production and oxidative stress, respectively. As shown in Fig. 6A, the plasma NO_x levels were increased in SHR-cp compared with WKY at both 18 and 23 weeks of age, and a tendency toward increasing plasma NO_x levels was observed in SHR. The plasma TBARS levels were markedly increased in SHR-cp compared with SHR and WKY at both 18 and 23 weeks of age (Fig. 6B).

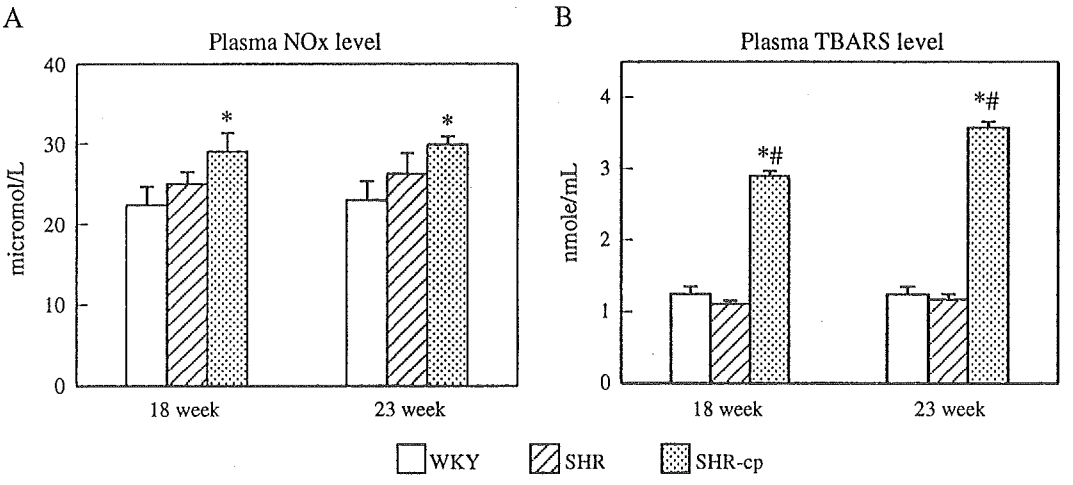

Fig. 6. Plasma levels of NO_x (NO_2 plus NO_3) (A) and TBARS (B) in SHR-cp, SHR and WKY at 18 and 23 weeks of age. Results are expressed as the mean±S.E.M. ($n=6$ for each group). $^*P<0.05$, as compared with the WKY, $^{\#}P<0.05$, as compared with the SHR.

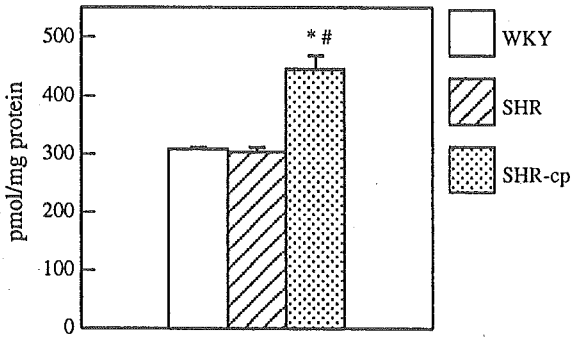

Fig. 7. Contents of 3-nitrotyrosine in aortas from SHR-cp, SHR and WKY at 20 weeks of age. Results are expressed as the mean ± S.E.M. ($n=3$ for each group). $^{*}P<0.05$, as compared with the WKY, $^{\#}P<0.05$, as compared with the SHR.

Protein levels of iNOS in aortas

The protein expression of iNOS was not detected in the aortas of not only WKY but also SHR-cp and SHR at 20 weeks of age (data not shown).

Contents of 3-nitrotyrosine in aortas

Peroxynitrite is a reactive oxidant species produced from NO and superoxide anion, and its involvement is evidenced by formation of 3-nitrotyrosine. Therefore, we determined the 3-nitrotyrosine levels in aortas of three strains at 20 weeks of age as an index of oxidative stress in the vascular wall. The 3-nitrotyrosine levels in aortas of SHR-cp significantly increased compared with SHR and WKY (Fig. 7).

Discussion

The metabolic syndrome is characterized by the coexistence of atherogenic risk factors, including obesity, hyperinsulinemia, hyperglycemia, hyperlipidemia, or hypertension, combined with underlying insulin resistance. The SHR-cp has been reported to simultaneously display early-onset obesity, hyperinsulinemia, hyperglycemia, hyperlipidemia and hypertension (Nangaku et al., 2003), and we confirmed these characteristics in the present study. Thus, SHR-cp seems to be a suitable animal model of the metabolic syndrome. Endothelium-dependent relaxations have been reported to be impaired in the metabolic syndrome (Steinberg et al., 1996; Zanchi et al., 1995). In some case, reduced endothelium-independent relaxations occur simultaneously (Karagiannis et al., 2003). In the present study, we also demonstrated that in aortas from the SHR-cp as well as SHR, both endothelium-dependent and -independent relaxations are impaired with increasing age when compared with normotensive WKY. Furthermore, SHR-cp and SHR showed decreases in sGC expression and cGMP levels produced by sodium nitroprusside, an NO donor, when compared with WKY, indicating the relaxation hypofunction of the vascular smooth muscle. A molecular target downstream of cGMP is likely to be involved in the vascular dysfunction, because the membrane-permeable stable analog of cGMP-induced relaxation showed a tendency to decrease in the SHR-cp with ageing. However, only at 18 weeks of age, SHR-cp but not SHR showed about the same relaxation response and a significant increase in cGMP level produced by acetylcholine, and significantly enhanced eNOS expression in comparison with WKY. At the same time, a significant increase in plasma levels of NO_x, as an index of generalized NO production, was also exhibited in SHR-cp. These changes with the exception of the relaxation response continued until 23 weeks of age. On the other hand, expression of the inducible NOS isoform was not observed in aortas of SHR-cp. Our findings indicate that in the aortas of SHR-cp, NO production in the endothelium considerably increases, although the NO-mediated relaxation response in the smooth muscle cells is impaired. The increased NO production probably antagonizes the decreased NO-mediated relaxation response at 18 weeks of age, and with aging, impairment of vascular reactivity to NO may greatly exceed the function of the endothelium.

Obese Zucker rats also spontaneously display obesity, hyperlipidemia and hyperinsulinemia, and the aged animals develop hypertension. Impairment of endothelium-dependent relaxations has been reported in the mesenteric and basilar artery of an animal model of the metabolic syndrome, obese Zucker rats, compared with age-matched lean heterozygous and control Zucker rats (Zanchi et al., 1995; Karagiannis et al., 2003). On the other hand, as demonstrated in the present study, considerably enhanced endothelium-dependent relaxation in response to acetylcholine and carbacol has been reported in the aorta of obese Zucker rats compared to the lean littermates (Auguet et al., 1989; Subramanian and MacLeod, 2003). The JCR:LA-corpulent rats have the *cp* gene, as well as SHR-cp, and animals homozygous for the *cp* gene develop obesity, hyperlipidemia, and hyperinsulinemia, but are normotensive. In the aorta from the JCR:LA-corpulent rats, both endothelium-dependent and -independent relaxations are not dramatically different compared to those in the lean rats at 7–24 weeks of age (Brunner et al., 2000; McKendrick et al., 1998; McNamee et al., 1994). Thus, alteration of vascular responsiveness may differ with animal species or age, and enhancement of endothelial NO production in SHR-cp can be enough to develop in parallel with the onset of the metabolic syndrome.

At present, the mechanisms for enhancement of endothelial NO production in SHR-cp remain unclear. eNOS is a constitutively expressed enzyme, but its expression is continually regulated by a number of biophysical, biochemical, and hormonal stimuli, both under physiological and pathophysiological conditions (Li et al., 2002a). For instance, insulin and leptin have been shown to stimulate NO release in endothelial cells via the activation or an increase in protein expression of eNOS (Li et al., 2002b; Vecchione et al., 2002). A marked increase in the plasma levels of these hormones is already known in SHR-cp (Nangaku et al., 2003) and SHR/N-cp, the original strain of SHR-cp (Velasque et al., 2001). Besides hormonal stimuli, upregulation of eNOS from increased oxidative stress has also been reported for various pathologic conditions, including hypertension, hyperlipidemia and diabetes (Taniyama and Griendling, 2003; Li et al., 2002b). The present study showed a dramatic increase in the plasma lipid

peroxide levels in SHR-cp compared with SHR as well as WKY. Furthermore, peroxynitrite, produced from NO and superoxide anion, is assumed to be present in the aortas of SHR-cp. Thus, it seems likely that eNOS upregulation observed in SHR-cp is caused by increased oxidative stress, probably resulting from the concomitant disease. This finding leads to the hypothesis that enhanced NO production may be a compensatory response to increases in oxidative stress as well as insulin and leptin levels in the metabolic syndrome. On the other hand, paradoxically, it is known that upregulation of eNOS expression causes the release of other products such as superoxide anion instead of NO, i.e. due to uncoupling of eNOS (Münzel et al., 2000). The expression pattern of eNOS depends on the stage, severity, and duration of the diseases, including hypercholesterolemia, atherosclerosis, hyperglycemia or diabetes (Li et al., 2002b). On the other hand, increased eNOS protein expression has been shown in the aorta from SHR both before and after the onset of hypertension (Vaziri et al., 1998). It remains unclear whether upregulation of the vascular NO production in aortas of SHR-cp depends on the genetic background of the strain. Further studies are needed to clarify the mechanism underlying enhanced NO production and the effect on the arterial wall.

Impairment of endothelium-independent relaxation response in the aortas of SHR-cp may, in part, result from hypertension, because a similar dysfunction was found in SHR. The vasodilator dysfunction in SHR-cp was observed despite unchanged sGC protein levels, suggesting that decreased sGC activity may precede reduction of its protein levels. With increasing age, SHR show progressive decreases in smooth muscle cGMP levels (Shirasaki et al., 1988) and sGC expression and activity (Kojda et al., 1998; Ruetten et al., 1999). These suggest that downregulation of the sGC/cGMP pathway also occurs in aortas of SHR-cp, possibly secondary to hypertension. The modulatory pathways of sGC expression and functionality have been shown, e.g. the long-term effects of NO itself on sGC or cross-talk regulation by cAMP (Andreopoulos and Papapetropoulos, 2000). Cyclic AMP, which is a product of cyclooxygenase-2 pathway, may play an important role in regulation of the NO/cGMP pathway under inflammatory conditions. On the other hand, NO is known to not only act as a stimulator of sGC but also has the potential to downregulate sGC expression (Andreopoulos and Papapetropoulos, 2000). We have recently proposed that downregulation of sGC is compensated by upregulation of eNOS, i.e., cross-talk occurs between the eNOS and sGC signaling pathways (Kagota et al., 2004). Thus, the possibility remains that the enhanced NO production in SHR-cp is partly involved in the downregulation of sGC activity or expression. Furthermore, it is noteworthy that increased oxidative stress downregulates the sGC protein expression (Mollnau et al., 2002; Courtois et al., 2003). Further studies, such as using drugs to modify blood pressure, are necessary to demonstrate that changes in the sGC/cGMP pathway are simply due to the increase of blood pressure.

In conclusion, the present study has demonstrated that in aortas of SHR-cp, a temporary increase in NO production from the endothelium occurs, although the NO/sGC/cGMP pathway in the smooth muscle cells is impaired. The upregulation of endothelial NO production may result from a variety of factors, including increases in oxidative stress as well as insulin in the metabolic syndrome, and may be a compensatory response to the decreased relaxation function in the vascular smooth muscle. On the other hand, dysfunction of the NO-mediated relaxations may be due to a decrease in sGC activity and expression, probably resulting from hypertension.

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REVIEW

Physicochemical and Immunological Research to Reduce the Dental Caries Epidemic – A Paradigm Shift in the Role of a Caries Vaccine

Hiroaki Takeuchi and Nobuhiro Hanada §

Department of Oral Health, National Institute of Public Health
(Chief : Nobuhiro Hanada)
2-3-6 Minami, Wako, Saitama 351-0197, Japan

Key words : mutans streptococci／caries epidemic／dental drug delivery system (3DS)／biofilm

Abstract : A previous report has revealed that dental caries is an infectious disease caused by a localized infection with specific oral streptococci. Dental-caries-associated oral streptococci are called the mutans streptococci (MS), with *Streptococcus mutans* and *Streptococcus sobrinus* being the most prevalent caries-associated organisms in humans. When searching for a control method against caries, effective measures are required at each of the following three phases of infectious disease : (1) eliminating the source of contagion, (2) blocking the infection route, and (3) lowering host susceptibility. In order to eliminate the source of contagion, we conducted a follow-up clinical test using a passive immunization method in which teeth were coated with anti-*S. mutans* specific antibodies. A subsidiary finding was made during the course of the test, in that physicochemical control methods were more effective than specific antibodies. Based on this finding, we have adopted a new caries-control method designated as the dental drug delivery system (3DS), and we have released an associated clinical manual (3DS unit).

In order to effectively eliminate MS from the oral cavity, however, we need to inhibit its initial adhesion as well as eliminating the source of contagion. Antibodies may be effective in the prevention of recolonization of MS.

Introduction

Mutans streptococci (MS), such as *Streptococcus mutans* and *S. sobrinus*, play an important role in the incidence of the dental caries epidemic. A previous report[1] has revealed that dental caries is an infectious disease caused by a localized infection with MS. The preventive process of dental caries can be categorized into three phases : (1) eliminating the source of contagion, (2) blocking the infection route, and (3) lowering host susceptibility. Therefore, a combination of effective measures for each of the three phases is required to develop a comprehensive dental caries-control method. Currently prevailing methods such as sucrose restriction and fluoride application are effective for phases (2) and (3) respectively. The use of a sugar substitute is useful in blocking the route of MS infection[2,3]. The use of fluoride to lower the critical pH of dental enamel also helps lower host susceptibility[4]. Eliminating the source of contagion (1) is at the least advanced stage of the dental caries control methods being used against the three phases of risk factors. Although clinical evaluations, such as research on the prevention of mother-to-infant transmission of MS[5] have been conducted, MS elimination measures have not been established in general prac-

§ Corresponding author
E-mail : nhanada @ niph.go.jp

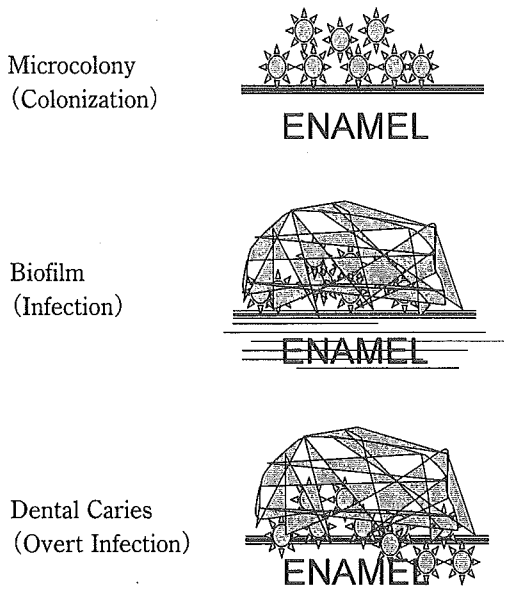

Fig. 1 Models of development of an MS biofilm
MS initially adhere to the dental pellicle via PAc (transmission). This is followed by microbial aggregation, leading to the formation of a microcolony (colonization). When sucrose intake is frequent, MS synthesize a water insoluble glucan catalyzed by GTF using sucrose as a substrate to form a cariogenic biofilm (infection). It strongly adheres to the tooth surface and aids in the storage of bacterial organic acids, which lead to dental caries (overt infection).

tice, even in developed countries.

MS initially adhere to the dental pellicle via a surface protein antigen (PAc)[6]. This is followed by microbial aggregation, leading to the formation of a microcolony. At this stage, MS elimination from the tooth surface is simple. When sucrose intake is frequent, MS synthesize a water insoluble glucan catalyzed by glucosyltransferase (GTF), using sucrose as a substrate to form a cariogenic biofilm[1]. This strongly adheres to the tooth surface and aids in the storage of bacterial metabolites (organic acids). This biofilm also aids in the drug resistance of MS.

At this stage, MS elimination from the tooth surface is complicated. (Fig. 1).

Several immunological control methods for MS have been reported[7,8]. However, whether the purpose of immunological control methods was to eliminate the source of contagion or to block the infection route is unclear. Immunological prevention methods for the dental caries epidemic can be classified into two categories : active immunity[9] that provides an antigen to the host, and passive immunity[10] that involves the direct incorporation of specific antibodies produced by other organisms. If there were a specific antibody the against the adhesive proteins such as PAc and GTF that is effective for immunological treatment in the oral cavity, it would be possible to inhibit the external adhesion of the MS, thereby blocking the route of infection. However, after MS form a microcolony, the effectiveness of a specific antibody appears questionable[11]. After MS has formed a biofilm on the tooth surface leading to a persistent infection, MS elimination cannot be accomplished even with a specific antibody coating. A biofilm infection is difficult to cure because of its low susceptibility to medication. Antibiotics and antibody molecules cannot effectively penetrate the biofilm. In particular, the susceptibility of biofilm-forming bacteria to antibiotics is extremely low due to their lowered metabolic activity within the biofilm[11]. Since full-mouth disinfection (FMD) advocated by Quirynen, *et al.*[12,13], using a nonspecific disinfectant, is more effective in killing commensal bacteria such as *Streptococcus mitis, S. oralis*, and *S. salivarius*, rather than the pathogenic biofilm, its use against dental caries is not possible. Therefore, once a biofilm has formed over the tooth surface, the elimination of the source of contagion is complicated[14].

Immunological Mechanisms for Bacterial Elimination

Cellular immunity does not function optimally in eliminating MS ; therefore, humoral immunity is the main route of acquired immunity. Humoral immunity against MS differs from the usual process of antigen phagocytosis where IgG is secreted into the blood. MS and the relevant antigen are taken into microfold cells (M cell) present in the mucous membrane, processed by antigen processing cells (APC), and recognized by T cells through MHC class II molecules. This causes B cells to recognize the antigen and multiply, and precursor cells to move into glandular tissues such as salivary glands, resulting in the production of

antigen-specific secretory immunoglobulin A (sIgA). Among the types of humoral immunity, mucous membrane immunity by the sIgA specific antibody from the salivary glands plays a particularly important role for dental caries prevention. The oral immune reaction mainly involves mucous membrane immunity, which primarily involves sIgA naturally present at 200 μg/mL in saliva.

There are two methods for immunologically eliminating MS : (1) an active immunity method which induces IgA (consisting of 60% of the immunoglobulins) by the direct presentation of the antigen derived from MS through a mucosal immunization, and (2) a passive immunity method, where a specific antibody against MS is produced externally and applied to the oral cavity.

The possible prevention of dental caries in humans using active immunization was first suggested in 1978[15,16]. A series of reports stated that the antibody titer of sIgA against *S. mutans* in saliva clearly increases when formalin-killed cells of *S. mutans* are orally administered[17,18]. Since then, the inhibition of *S. mutans* adhesion, the production of a GTF-inhibiting sIgA antibody by oral administration of killed cells[19] as well as adhesion inhibition by oral administration of GTF[20] have been reported. Childers, *et al.*[21] have also reported the production of inhibiting sIgA with GTF oral administration.

However, no case of eliminating MS by active immunity alone has thus far been reported. In 1998, Lehner's group[22] in England passively immunized a human oral cavity with Guy's 13 monoclonal antibody derived, genetically altered, plant-originated, anti-*S. mutans* sIgA. This study captured the attention of many researchers in the world, since it clearly indicated the elimination of *S. mutans*. The passive immunity method, wherein an effective specific antibody is applied externally, has low risks of side effects and is easy to administer due to its safety.

A Follow-up Clinical Test

Lehner's group concluded that passive immunity that involves the use of a specific antibody is essential for eliminating MS. To confirm this, we conducted a follow-up clinical test[23] in humans with regard to passive immunotherapy, using monoclonal antibodies (mAb) specific to *S. mutans*. Three types of anti *S. mutans* mAb (p126, P136 and KH5)[24,25] were used. The follow-up clinical test[23] was carried out by seven subjects who are engaged in research in this field. A crossover study design was adopted. The test consisted of a first trial in which saline was applied instead of antibody, and a second trial in which anti *S. mutans* mAb was applied. The test evaluated the numbers and ratios of MS in the saliva of each subject. Prior to coating the tooth surfaces with antibodies, we performed a biofilm destruction and chemical elimination procedure identical to that used in the study by Lehner's group[22]. These pre-treatment procedures included professional mechanical tooth cleaning (PMTC) and local application of a disinfectant on the tooth surfaces using custom trays. Reasons for performing pre-treatment procedures are : (1) to minimize the amount of antigen (*S. mutans* epitope), which tends to exist in overwhelming proportions in comparison with the amount of antibody, and (2) to convert the antigen into a planktonic state through the mechanical destruction of the biofilm with PMTC, since the antibody molecules themselves cannot couple with bacteria in the biofilm[11]. The mAb application (passive immunity) protocol used in our follow-up clinical test included the use of mAb P126 and P136[24] that inhibit the activity of GTF. The test also included the use of mAb KH5[25] against the PAc molecules associated with the recolonization of MS to tooth surfaces. It is recognized that KH5 is effective for an over 60% adhesion inhibition of MS on rat tooth surfaces, and that P126 and P136 are effective for inhibition of dental caries in rats[24]. To avoid contamination by mouse hybridoma-originated unknown virus pathogens, safety precautions were taken by treating the antibodies with gamma ray irradiation following a multi-step purification process. Three types of antibodies, anti-PAc antibody and two types of anti-GTF mAb, were mixed at a ratio of KH5 : P136 : P126 = 1 : 1 : 1 (V/V) (Table 1) and dissolved in a PBS buffer to yield a final concentration of 1.55 mg/mL, anticipating a synergetic effect by differing inhibition mechanisms. In this experiment, a custom